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(54) **HUMAN NEURAL STEM CELLS EXPRESSING HUMAN CHOLINE ACETYLTRANSFERASE, AND USE THEREOF**

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CPC *A61K 35/30* (2013.01); *A61K 48/005* (2013.01); *C12N 5/0623* (2013.01); *C12N 9/1029* (2013.01); *C12Y 203/01006* (2013.01); *C12N 2510/00* (2013.01)

(58) **Field of Classification Search**
CPC A61K 38/43
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,656,743	B2 *	12/2003	Huson et al.	435/193
2004/0138117	A1 *	7/2004	Hudson et al.	514/12
2007/0054278	A1 *	3/2007	Cargill	435/6

FOREIGN PATENT DOCUMENTS

WO	WO 2006/102902	10/2006	....	C12N 5/00
WO	WO 2006102902	A1 *	10/2006	

OTHER PUBLICATIONS

Xuan et al. "Effects of engrafted neural stem cells in Alzheimer's disease rats." Neurosci Lett. Jan. 30, 2009;450(2):167-71.*

Kim et al. "Brain transplantation of human neural stem cells transduced with tyrosine hydroxylase and GTP cyclohydrolase 1 provides functional improvement in animal models of Parkinson disease." Neuropathology. Apr. 2006;26(2):129-40.*

Lee et al. "Human neural stem cells over-expressing VEGF provide neuroprotection, angiogenesis and functional recovery in mouse stroke model." PLoS One. Jan. 17, 2007;2(1):e156.*

Zhong et al., "Discrete Acetylcholine Release from Neuroblastoma or Hybrid Cells Overexpressing Choline Acetyltransferase into the Neuromuscular Synaptic Cleft," *Neuroscience Research* 22: 81-88, 1995.

Zhong et al., "Overexpression of Choline Acetyltransferase Reconstitutes Discrete Acetylcholine Release in Some but Not All Synapse Formation-Defective Neuroblastoma Cells," *J. Physiology* 89: 137-145, 1995.

International Search Report for PCT/KR2011/004886, mailed Feb. 10, 2012 (4 pages).

Kim, Seung U., *"Human neural stem cells genetically modified for brain repair in neurological disorders"*, Neuropathology 2004; 24, 159-171.

Lee, Hong J., et al., *"Human Neural Stem Cells Genetically Modified to Express Human Nerve Growth Factor (NGF) Gene Restore Cognition in the Mouse With Ibotenic Acid-Induced Cognitive Dysfunction"*, Cell Transplantation, vol. 21, pp. 2487-2496, 2012, 10 pages.

Park, Dongsun, et al., *"Human Neural Stem Cells Overexpressing Choline Acetyltransferase Restore Cognitive Function of Kainic Acid-Induced Learning and Memory Deficit Animals"*, Cell Transplantation, vol. 21, pp. 365-371, 2012.

Park, Dongsun, et al., *"Human neural stem cells over-expressing choline acetyltransferase restore cognition in rat model of cognitive dysfunction"*, Experimental Neurology 234 (2012) 521-526.

Park, Dongsun, et al., *"Improvement of cognitive function and physical activity of aging mice by human neural stem cells over-expressing choline acetyltransferase"*, Neurobiology of Aging 34 (2013) 2639-2646.

(Continued)

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(57) **ABSTRACT**

The present invention relates to human neural stem cells (NSCs) transfected with a vector comprising a polynucleotide encoding human choline acetyltransferase (ChAT) and thereby stably expressing the human ChAT, a composition for treating Alzheimer disease or for improving a cognitive disorder comprising the human NSCs expressing a human ChAT. The present human NSCs genetically modified to express human ChAT, when transplanted into the brain of the animal AD model, successfully integrated into the host tissues and differentiated into the normal neuronal cells or glial cells. The instant genetically modified human NSCs stably express ChAT in the brain tissue of AD animal and thereby restore the acetylcholine level, and learning and memory function comparable to normal animal. The present genetically modified human NSCs expressing ChAT can be used for the treatment of AD as well as cognitive disorders due to other brain diseases and aging.

6 Claims, 4 Drawing Sheets

(56)   **References Cited**

OTHER PUBLICATIONS

Yasuhara, Takao; "*Transplantation of Human Neural Stem Cells Exerts Neuroprotection in a Rat Model of Parkinson's Disease*", The Journal of Neuroscience, Nov. 29, 2006; 26(48):12497-12511.

Lee, Hong J., et al., "*Brain Transplantation of Immortalized Human Neural Stem Cells Promotes Functional Recovery in Mouse Intracerebral Hemorrhage Stroke Model*"; Stem Cells 2007;25:1204-1212.

Long, A.G. Xuan, et al., "*BDNF improves the effects of neural stem cells on the rat model of Alzheimer's disease with unilateral lesion of fimbriafornix*", Neuroscience Letters 440 (2008) 331-335.

Blurton-Jones, Mathew, et al., "*Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease*", PNAS, Aug. 11, 2009, vol. 106 No. 32, 6 pages.
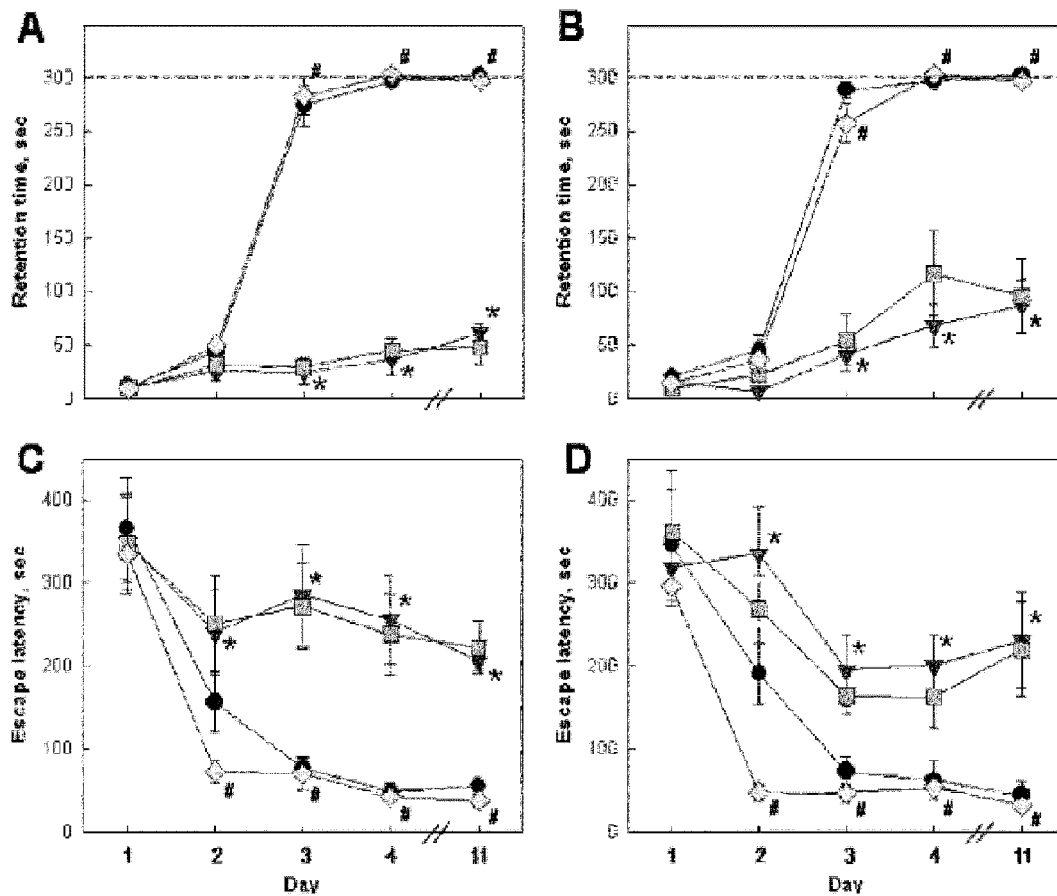
Hwang, Dong H., et al., "*Transplantation of human neural stem cells transduced with Olig2 transcription factor improves locomotor recovery and enhances myelination in the white matter of rat spinal cord following contusive injury*", BMC Neuroscience 2009, 10:117 doi:10.1186/1471-2202-10-117, 16 pages.

* cited by examiner

Fig. 1
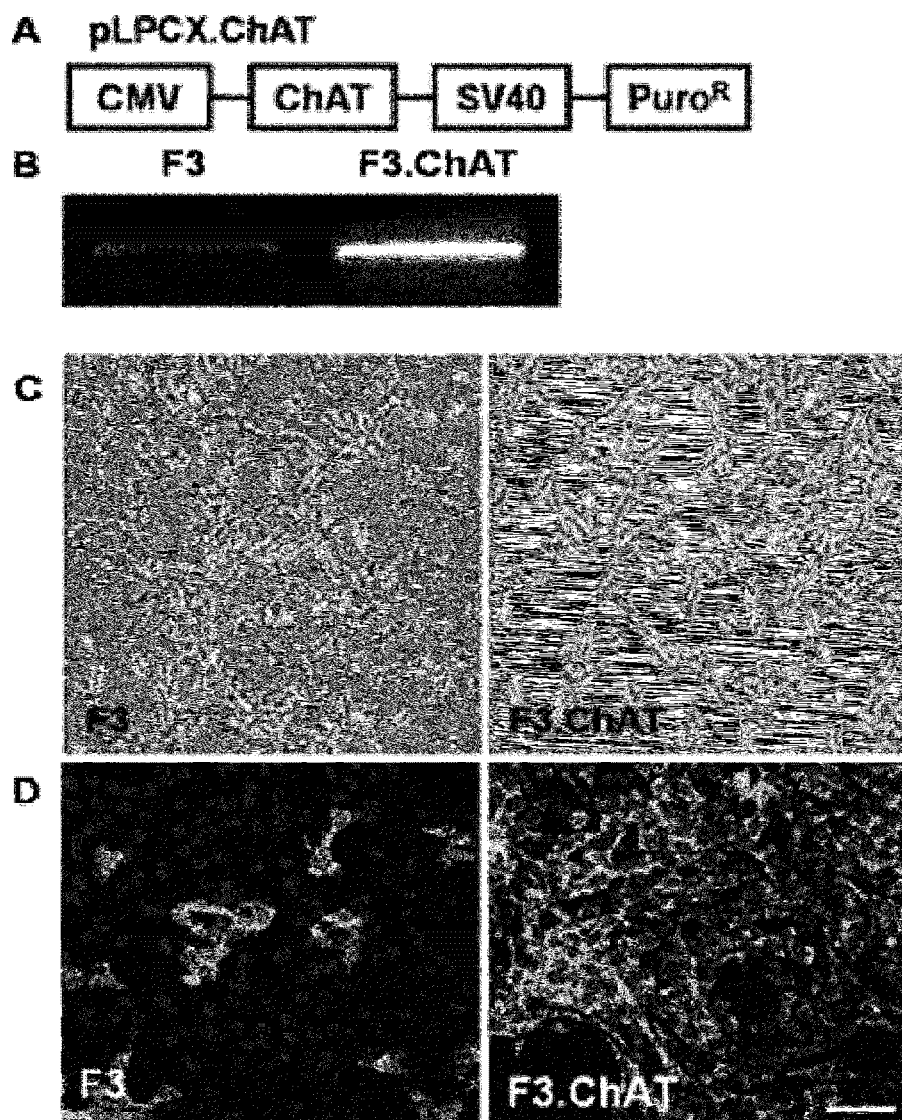
Fig. 2
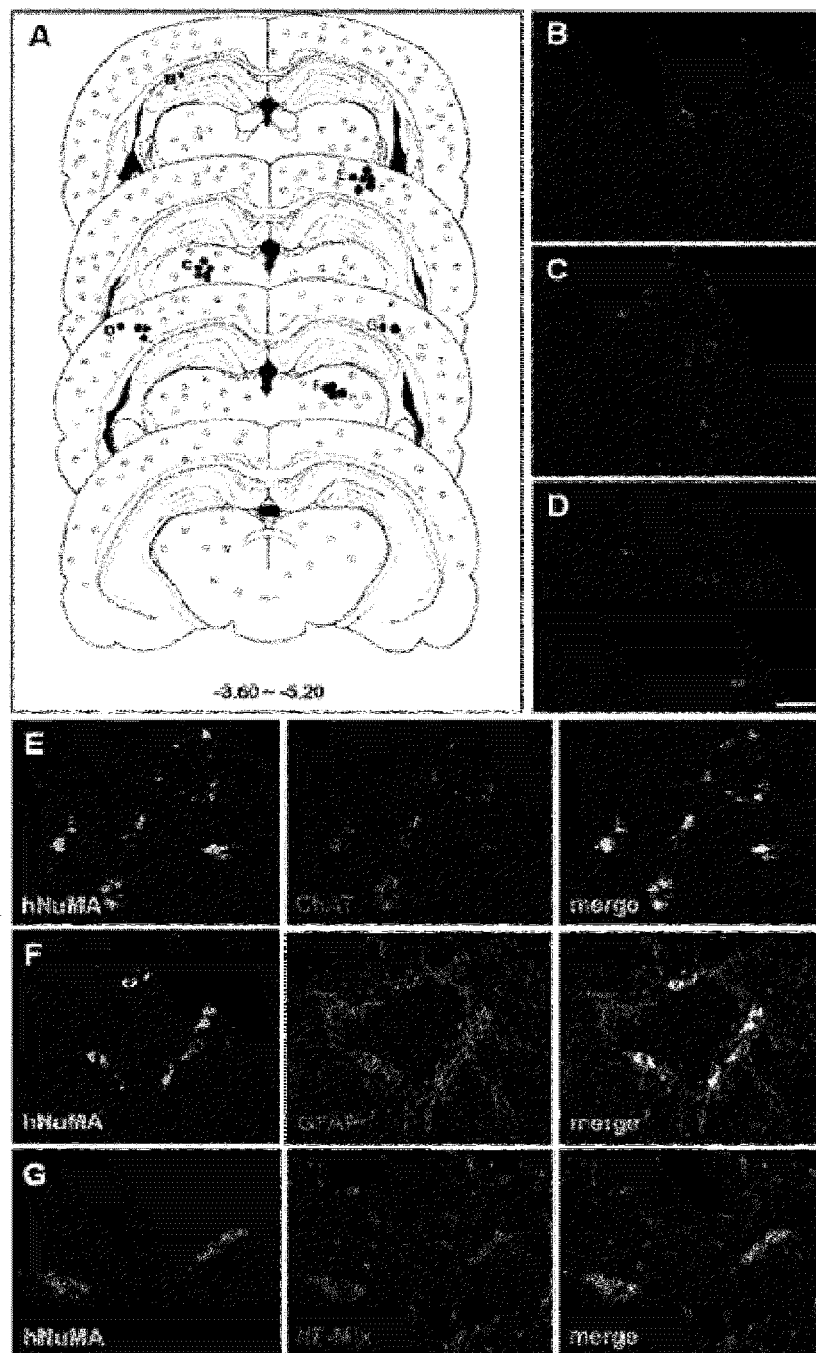
Fig. 3
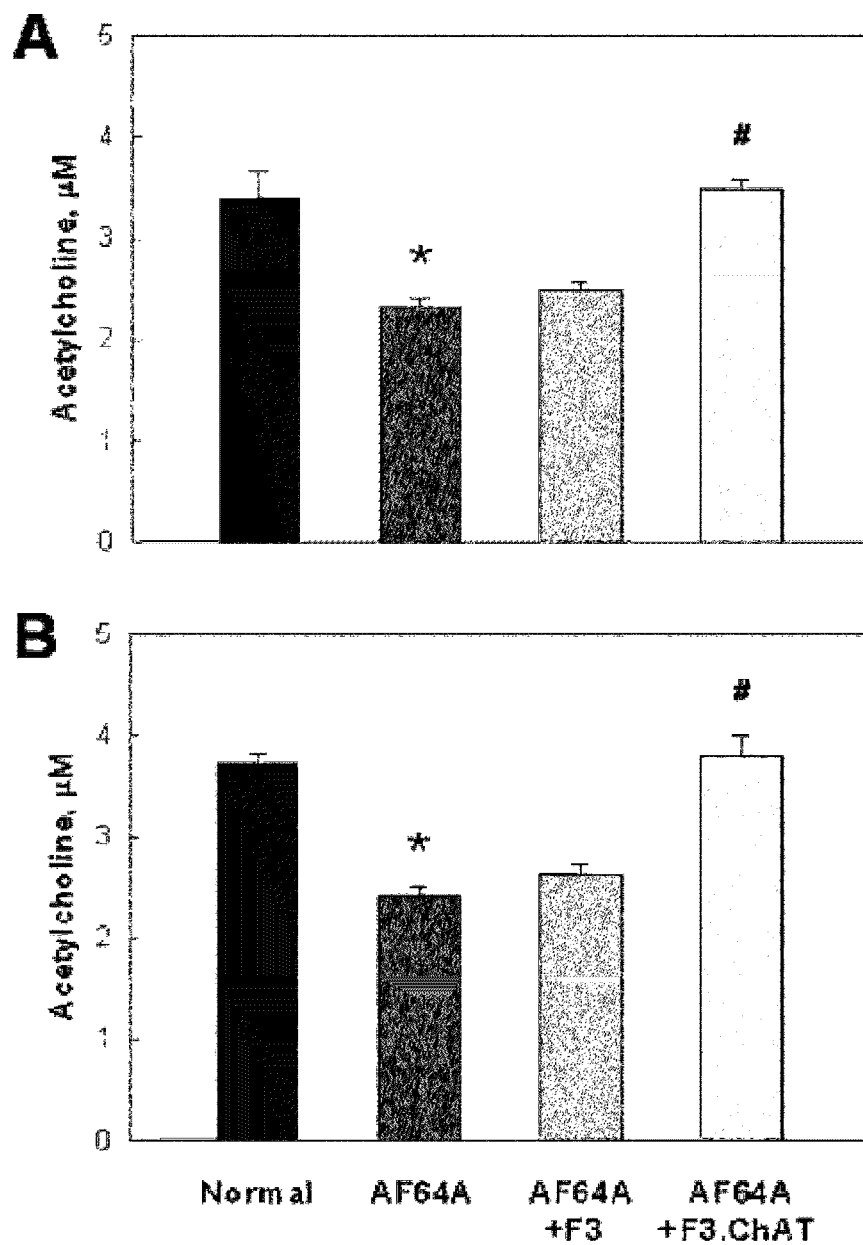
Fig. 4

HUMAN NEURAL STEM CELLS EXPRESSING HUMAN CHOLINE ACETYLTRANSFERASE, AND USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is the U.S. national stage filing under 35 U.S.C. §371 of International Application No. PCT/KR2011/004886, filed Jul. 4, 2011, which claims priority from Korean Patent Application No. 10-2010-0098234, filed on Oct. 08, 2010, in the Korean Intellectual Property Office, the disclosure of which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to human neural stem cells (NSCs) transfected with a vector comprising a polynucleotide encoding human choline acetyltransferase (ChAT) and thereby stably expressing the human ChAT. More specifically, the present invention relates to a pharmaceutical composition for treating Alzheimer disease or for improving a cognitive disorder comprising human NSCs expressing a human ChAT.

DESCRIPTION OF THE RELATED ART

Alzheimer disease (AD) is one of the most devastating neurodegenerative diseases, which is characterized by deficit of cognitive functions including learning and memory loss (1). In AD patients, the dysfunction of presynaptic cholinergic system is one of the primary causes of cognitive disorders (2, 3), in which decreased activity of enzyme choline acetyltransferase (ChAT) responsible for acetylcholine (Ach) synthesis is observed (4, 5). To date, AD therapy is largely based on small molecule designed to increase ACh concentration by inhibiting acetylcholinesterase (AChE), an ACh-degrading enzyme (5, 6). Since therapy with the drugs is only palliative without potential protection against progressive tissue destruction, there is substantial need for effective therapies for patients with AD, and stem cell-based therapeutic approach targeting AD should fulfill this requirement.

For many neurological disorders, studies have begun to examine stem cell-based therapies as a novel strategy to treat disorders such as Parkinson disease (PD), Huntington disease, amyotrophic lateral sclerosis (ALS), stroke and spinal cord injury (7-9). In contrast to a transient improvement of body function by pharmaceuticals, it is expected that stem cells can prevent or delay host cell death and restore injured tissue (7-10). Notably, we have previously demonstrated that transplantation of human neural stem cells (NSCs) expressing diverse functional genes, especially encoding growth factors, preserves host cells and recovered body function in animal models of stroke, PD, ALS, and spinal cord injury (11-14). Immortalized human NSCs have emerged as highly-effective source of cells for genetic manipulation and gene transfer into the central nervous system (CNS) ex vivo; genetically modified NSCs survive, integrate into host tissues and differentiate into both neurons and glial cells after transplantation into intact or damaged brain (9).

Throughout this application, various publications and patents are referred and citations are provided in parentheses. The disclosures of these publications and patents in their entities are hereby incorporated by references into this application in order to fully describe this invention and the state of the art to which this invention pertains.

DETAILED DESCRIPTION OF THIS INVENTION

Technical Purposes of This Invention

The present inventors have made intensive studies to develop a stem cell-based therapeutics for alleviating cognitive disorders such as learning or memory dysfunction in Alzheimer disease patients who lack acetylcholine. The instant inventors established human neuronal stem cell (NSC) lines over-expressing human choline acetyltransferase (ChAT) and transplanted the prepared human NSCs into the brain of animal of Alzheimer disease model. As a result, the inventors demonstrated that the transplanted human NSCs over-expressing human ChAT stably differentiates into normal neuron cells in the brain of the animal and successfully recover the learning and memory function of the Alzheimer disease animal.

Accordingly, it is an object of this invention to provide human neural stem cells (NSCs) expressing human choline acetyltransferase (ChAT).

It is another object of this invention to provide a pharmaceutical composition for treating Alzheimer disease comprising human NSCs expressing human ChAT.

It is still another object of this invention to provide a pharmaceutical composition for improving a cognitive disorder comprising human NSCs expressing human ChAT.

It is still another object of this invention to provide a method of treating Alzheimer disease comprising administering to a subject suffering from Alzheimer disease a pharmaceutically effective amount of human NSCs expressing human ChAT.

It is still another object of this invention to provide a method of improving a cognitive disorder comprising administering to a subject suffering from cognitive disorder a pharmaceutically effective amount of human NSCs expressing human ChAT.

It is still another object of this invention to provide a pharmaceutical composition comprising human NSCs expressing human ChAT for use in a method of treating Alzheimer disease.

It is still another object of this invention to provide a pharmaceutical composition comprising human NSCs expressing human ChAT for use in a method of treating a cognitive disorder.

Other objects and advantages of the present invention will become apparent from the following detailed description together with the appended claims and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 represents F3.ChAT human neural stem cell line generated by infecting F3 human neural stem cells with retroviral vector encoding human ChAT gene. Panel (A) depicts the structure of the retroviral vector. The plasmid, pLPCX .ChAT contains the full-length human ChAT cDNA. Panel (B) shows the results of RT-PCR analysis of ChAT mRNA expression. Panel (C) displays the morphology of F3 (left) and F3.ChAT (right) cells. Panel (D) shows ChAT protein (green-colored) in F3 (left) and F3.ChAT (right) cells detected by the immunocytochemical method. Scale bar, 50 μm. An immortalized NSC line, HB1.F3 (F3), was established from primary cultures of a 15-week gestational human fetal brain by infecting with a retroviral vector encoding v-myc oncogene (9, 11, 28). The F3 NSC line was infected with a retrovirus encoding human ChAT gene. Plasmid pLPCX.ChAT containing the full-length human ChAT cDNA was used. PG13 mouse packaging cell line was transfected

with pLPCX.ChAT vector using SuperFect reagent, and stable PG13 cell line were selected using 5 μg/ml puromycin for 7 days. Replication incompetent retroviral vector collected from PG13.ChAT cells was used for infection of F3 human NSCs and puromycin-resistant F3.ChAT clones were isolated, and one of the clones F3.ChAT.D2 was expanded, and used for the transplantation. For analysis of ChAT mRNA expression, RT-PCR was performed. For demonstration of ChAT protein, cells were incubated with primary antibody specific for human ChAT followed by fluorescent 2nd antibody, and viewed under a laser-scanning confocal microscope.

FIG. 2 shows the feature of the transplanted human F3.ChAT cells in the rat brain. Panels (A)-(D) represent the distribution of human F3.ChAT cells (identified by hNuMA-positive reaction) in the rat brain 9 weeks post-transplantation (1×10^6 cells/rat) in AF64A-injected rats. Panel (E) shows the ChAT protein production in the rat brain 9 weeks post-transplantation (1×10^6 cells/rat) in AF64A-injected rats. Panels (F) and (G) is the feature of the differentiation of the transplanted human F3.ChAT cells into astrocytes (GFAP-stained, F) and neurons (NF-Mix-stained, G) of human (hNuMA-stained) F3.ChAT cells 9 weeks post-transplantation. Scale bar, 50 μm.

FIG. 3 shows the acetylcholine (ACh) concentration in rat cerebrospinal fluid (CSF) (panel A, 5 weeks; panel B, 9 weeks post-transplantation) post-transplantation of F3 or F3.ChAT cells (1×10^6 cells/rat) following 2-week pretreatment with AF64A (3 nmol/rat). ACh concentration in CSF was measured with the Amplex Red acetylcholine/acetylcholinesterase assay kit (Molecular Probes). In this assay, ACh is hydrolyzed by AChE to release choline, which is then oxidized by choline oxidase to betaine and H_2O_2. H_2O_2 interacts with Amplex Red (7-dihydroxyphenoxazine) in the presence of horseradish peroxidase to generate the highly fluorescent resorufin. The resulting fluorescence was measured in a fluorescence microplate reader using excitation in the range of 530-560 nm and emission at ~590 nm.

FIG. 4 shows the experimental results of the passive avoidance (panels A and B) and water-maze (panels C and D) performances 4-5 weeks (panels A and C) and 8-9 weeks (panels B and D) post-transplantation of F3 or F3.ChAT cells (1×10^6 cells/rat) following 2-week pretreatment with AF64A (3 nmol/rat). ●, normal control; ▼, AF64A alone administration; ■, AF64A+F3; ♦, AF64A+F3.ChAT. On passive avoidance trials, electric shock (1 mA for 2 sec) was delivered when rats entered the dark compartment from the light room through a guillotine door. The latency time of stay in the light room from light-on was recorded. The end-point was set at 300 sec, denoting full acquisition of memory. On water-maze tests, rats were subjected to a performance consisted of 3 trials a day for 4 consecutive days, followed by 5th performance for further confirmation 1 week after the daily 4 performances at 4 or 8 weeks. The mean escape latency onto the platform was calculated.

DETAILED DESCRIPTION OF THIS INVENTION

In one aspect of this invention, there is provided human neural stem cells (NSCs) transfected with a vector comprising a polynucleotide encoding human choline acetyltransferase (ChAT) and thereby stably expressing human choline acetyltransferase.

The term "choline acetyltransferase (ChAT)" used herein refers to an enzyme responsible for the synthesis of neurotransmitter acetylcholine by combining acetyl-CoA with choline. The acetylcholine is produced in the body of neuron

and moved into the terminal of the neuron through the axoplasmic flow. The choline acetyltransferase is encoded by the ChAT gene in human [Strauss, W. L., Kemper R. R., Jayakar, P. et al., Genomics 9, 396-398 (1991)].

According to a preferred embodiment, the choline acetyltransferase comprises the amino acid sequence of SEQ ID NO:1 (GenBank Accession No. NP_065574).

The term "neural stem cells (NSCs)" used herein refers to cells that can continuously self-renew and have the multipotential to generate neurons (neuronal cells), astrocytes, and oligodendrocytes constituting the central nervous system.

According to a preferred embodiment, the neural stem cells of this invention are primary cultured neural stem cells or genetically modified immortalized neural stem cells.

The human neural stem cells of the present invention can be primary cultured neural stem cells isolated from various tissue of the human. For example, neural stem cells isolated from a human adult brain or fetal brain, neural stem cells induced from hematopoietic stem cells in a human cord blood or bone marrow, or neural stem cells derived from a human embryo can be used for the present invention.

The human neural stem cells of the present invention can be genetically modified immortalized one. For example, immortalized neural stem cells established by introducing a retroviral vector containing v-myc oncogene can be used for the present invention.

Human neural stem cells can be identified by positive staining of the stem cell marker of Nestin.

The detailed method for preparing neural stem cells is described in U.S. Pat. No. 5,654,183 and is incorporated herein as a reference. Human neural stem cells can be cultured in the media in the presence of growth factors such as bFGF (basic fibroblast growth factor), EGF (epidermal growth factor), or FGF (fibroblast growth factor) with a suitable concentration, for example 5-100 ng/ml.

The human neural stem cells of the present invention are genetically modified cells which have been transfected with a vector comprising human choline acetyltransferase (ChAT).

As demonstrated by the following specific examples of the present invention, human choline acetyltransferase (ChAT), which is introduced into the human neural stem cells (NSCs) through the viral vector, is stably expressed in the host cells. Furthermore, the present human NCSs expressing the human ChAT, when transplanted into the brain of animal of Alzheimer disease model, stably survive, integrate into the host brain tissues, and differentiate into neurons and astrocytes, and eventually restore cognitive functions comparable to the normal animal by elevating the acetylcholine level.

The vector that can be used for the introduction of human ChAT gene into the human NSCs in the present invention preferably includes without limitation (i) adenoviral vector, (ii) retroviral vector, (iii) adeno-associated viral vector, (iv) herpes simplex viral vector, (v) SV 40 vector, (vi) polyoma viral vector, (vii) papilloma viral vector, (viii) picornaviral vector, (ix) vaccinia viral vector, and (x) helper-dependent adenoviral vector.

The replication origin that can be used in the present vector is preferably operable in the eukaryotic cells, and more preferably comprises without limitation f1 replication origin, SV40 replication origin, pMB1 replication origin, Adeno replication origin, AAV replication origin, and BBV replication origin.

The promoter that can be used in the present vector preferably includes without limitation the promoters derived from the genome of mammalian cells (e.g. metallothionein promoter) or derived from mammalian viruses (e.g. the adenovirus late promoter, the vaccinia virus 7.5K promoter, SV40

promoter, cytomegalovirus promoter, and HSV tk promoter). The polyadenylation sequence as a transcription termination signal sequence that can be used in the instant vector preferably includes without limitation SV40-derived polyadenylation sequence or BGH polyA sequence.

According to this invention, the vector may include a suitable selective marker, preferably includes antibiotic resistance gene, such as the resistance genes for ampicillin, gentamicin, carbenicillin, chloramphenicol, streptomycin, kanamycin, geneticin, neomycin, puromycin, or tetracycline.

According to the most preferred embodiment of this invention, the structure of the vector for expressing a human choline acetyltransferase is depicted in the panel A of FIG. 1.

The introduction of the vector into human neural stem cells can be performed through various methods known to those skilled in the art, for example, microinjection [Capecchi, M. R., Cell, 22:479 (1980)], calcium phosphate co-precipitation [Graham, F. L. et al., Virology, 52:456 (1973)], electroporation [Neumann, E. et al., EMBO J., 1:841 (1982)], liposome-mediated transfection [Wong, T. K. et al., Gene, 10:87 (1980)], DEAE-dextran treatment [Gopal, Mol. Cell Biol., 5:1188-1190 (1985)], and particle bombardment [Yang et al., Proc. Natl. Acad. Sci., 87:9568-9572 (1990)].

The human neural stem cells that are transfected with the vector containing ChAT gene can be easily selected by utilizing the phenotype expressed by selection marker. For example, if the selection marker is the resistance gene for the particular antibiotic, the transformed human NSCs can be selected by cultivating the host cells in the media containing the antibiotic.

In another aspect of this invention, there is provided a pharmaceutical composition for treating Alzheimer disease comprising human neural stem cells transfected with a vector comprising a polynucleotide encoding human choline acetyltransferase and thereby stably expressing human choline acetyltransferase.

The pharmaceutical composition according to this invention may typically be prepared as injectables, either as liquid solutions or suspensions containing the cells. Pharmaceutical forms suitable for injection include sterile aqueous solutions or dispersions for extemporaneous preparation of the solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that is feasible for injection.

The pharmaceutical composition may include pharmaceutically acceptable carrier compatible with human neural stem cells. The term "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The pharmaceutically acceptable carrier includes any solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained by the use of a coating such as lecithin. The prevention of the contamination of microorganisms can be accomplished by the use of various antibacterial and antifungal agents such as parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, isotonic agents may be included, for example, sugars or sodium chloride. Prolonged adsorption of the injectable compositions can be brought about by the use in the composition of agents delaying absorption, for example, aluminum monostearate and gelatin. Sterile injectable solutions are prepared by incor-

porating the active agents in the required amount in the appropriate solvent with various other ingredients followed by filtered sterilization.

The pharmaceutical composition according to this invention is preferably administered parenterally, i.e., by intraperitoneal, subcutaneous, intramuscular, intravenous, intracerebroventricular, intraspinal or local administration. Most preferably, the pharmaceutical composition of this invention can be administered by direct injection into the brain region.

The pharmaceutical composition according to this invention may be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. A suitable dosage amount of the pharmaceutical composition of this invention may vary depending on the condition of the subject being treated. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluents first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, intradermal, intracerebroventricular, intraspinal and intraperitoneal administration. In this connection, carrier, formulation, and media that can be employed in the present composition are well known to those skilled in the art. See for example, "Remington's Pharmaceutical Sciences" 15th Edition.

In still another aspect of this invention, there is provided a composition for improving a cognitive disorder comprising human neural stem cells (NSCs) transfected with a vector comprising a polynucleotide encoding human choline acetyltransferase (ChAT) and thereby stably expressing human ChAT.

According to a preferred embodiment, the cognitive disorder is a disorder of learning function or memory function.

According to another preferred embodiment, the cognitive disorder is caused by the reduction of level of acetylcholine released in the brain.

According to still another preferred embodiment, the composition for improving a cognitive disorder is formulated as a form of pharmaceutical composition. Since the pharmaceutical composition for treating Alzheimer disease is described herein above and therefore the above descriptions can be adapted to the pharmaceutical composition for improving a cognitive disorder. Accordingly, the common descriptions between them are omitted in order to avoid undue redundancy leading to the complexity of this specification.

In still another aspect of this invention, there is provided a method of treating Alzheimer disease comprising administering to a subject suffering from Alzheimer disease a pharmaceutically effective amount of human neural stem cells (NSCs) transfected with a vector comprising a polynucleotide encoding human choline acetyltransferase (ChAT) and thereby stably expressing human ChAT.

In still another aspect of this invention, there is provided a method of treating a cognitive disorder comprising administering to a subject suffering from a cognitive disorder a pharmaceutically effective amount of human neural stem cells (NSCs) transfected with a vector comprising a polynucleotide encoding human choline acetyltransferase (ChAT) and thereby stably expressing human ChAT.

In still another aspect of this invention, there is provided a pharmaceutical composition comprising a human neural stem cells (NSCs) transfected with a vector comprising a polynucleotide encoding human choline acetyltransferase (ChAT) and thereby stably expressing human ChAT for use in a method of treating Alzheimer disease.

In still another aspect of this invention, there is provided a use of a human neural stem cells (NSCs) transfected with a

vector comprising a polynucleotide encoding human choline acetyltransferase (ChAT) and thereby stably expressing human ChAT for the manufacture of a medicament for use in treatment of Alzheimer disease.

In still another aspect of this invention, there is provided a pharmaceutical composition comprising a human neural stem cells (NSCs) transfected with a vector comprising a polynucleotide encoding human choline acetyltransferase (ChAT) and thereby stably expressing human ChAT for use in a method of treating a cognitive disorder.

In still another aspect of this invention, there is provided a use of a human neural stem cells (NSCs) transfected with a vector comprising a polynucleotide encoding human choline acetyltransferase (ChAT) and thereby stably expressing human ChAT for the manufacture of a medicament for use in treatment of a cognitive disorder.

Advantageous Effects

The present invention relates to human neural stem cells (NSCs) transfected with a vector comprising a polynucleotide encoding human choline acetyltransferase (ChAT) and thereby stably expressing the human ChAT. More specifically, the present invention relates to a pharmaceutical composition for treating Alzheimer disease or for improving a cognitive disorder comprising the human NSCs expressing a human ChAT. The features and advantages of the present invention will be summarized as follows:

(i) the present human NSCs genetically modified to express human ChAT, when transplanted into the brain of the animal AD model, successfully integrated into the host tissues and differentiated into the normal neuronal cells or glial cells.

(ii) the present genetically modified human NSCs stably express ChAT in the brain tissue transplanted and thereby restore the acetylcholine level comparable to the normal level.

(iii) the present genetically modified human NSCs expressing ChAT restore the cognitive functions including learning and memory functions in the animal AD model.

(iv) the present genetically modified human NSCs expressing ChAT can be used for the treatment of Alzheimer disease as well as cognitive disorders due to other brain diseases and aging.

The present invention will now be described in further detail by examples. It would be obvious to those skilled in the art that these examples are intended to be more concretely illustrative and the scope of the present invention as set forth in the appended claims is not limited to or by the examples.

EXAMPLES

Example 1

Human Choline Acetyltransferase (ChAT) Cloning and cDNA Constructs

Full length cDNA of human choline acetyltransferase (ChAT) was obtained by PCR from human small intestine Marathon-Ready cDNA (Clontech, Mountain View, Calif.) following the procedures previously described for rat peripheral type ChAT (29). Sequencing of plasmid DNA was determined with ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.).

Example 2

Human Neural Stem Cell (NSC) Lines

HB1.F3 (F3) human NSC line (8, 9, 11, 28) was infected with a retrovirus with pLPCX.ChAT containing the full-

length human ChAT cDNA, selected for puromycin resistance (13). F3.ChAT, ChAT-overexpressing clone, was isolated, and expression of ChAT was analyzed by RT-PCR and immunofluorescence microscopy.

Example 3

RT-PCR Analysis

Total RNA was extracted from NSC cultures using TRIzol (BRL, Gaithersburg, Md.). Complimentary DNA templates were prepared from 1 mg of total RNA primed with oligo dT primers using 400 U of Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, Wis.) followed by 25 PCR cycles, and RT-PCR products were separated electrophoretically on 1.2% agarose gel containing ethidium bromide. The primers used for the ChAT RT-PCR are following:

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                                    (SEQ ID NO: 2)
    Sense:         5'-CTGTGCCCCCTTCTAGAGC-3';

                                    (SEQ ID NO: 3)
    Antisense:     5'-CAAGGTTGGTGTCCCTGG-3'.
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Example 4

Immunocytochemistry of NSCs

F3.ChAT cells were plated on poly-L-lysine-coated Aclar plastic coverslips and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 5 min at room temperature. Fixed cultures were incubated with the primary antibody specific for human ChAT (1:100, rabbit polyclonal, Chemicon, Temecula, Calif.) for 24 hr at 4° C., followed by Alexa Fluor 488-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR) for 1 hr at room temperature (RT). Cells were counterstained with 4',6-diamino-2-phenylindole (DAPI, Sigma, St. Louis, Mo.) to identify cellular nuclei.

Example 5

AD Model and NSCs Transplantation

Male Sprague-Dawley rats (Orient-Bio, Seongnam, Korea) weighing between 220-230 g were anesthetized with enflurane and positioned in a stereotaxic frame. After incision of the skin and drilling a hole, freshly-prepared AF64A (RBI, Natick, Mass.) solution (3 nmol/3 μl/rat) (17, 20) was infused into the right ventricle, at the following stereotaxic coordinates from bregma: posterior 0.8 mm, lateral 1.5 mm, and ventral 4.0 mm, at a flow rate of 0.5 μl/min (17, 21). Two weeks later, F3 or F3·ChAT cells (1×10^6 cells/rat) were transplanted in rats showing loss of learning and memory functions (n=15/group) via intracerebroventricular (icy) injection at the same coordinates.

Example 6

Learning/Memory Testing

The rats were subjected to learning and memory function tests 4-5 weeks (n=8/group) or 8-9 weeks (n=7/group) after transplantation of the cells. The passive avoidance trials were performed once a day for consecutive 4 days and 5th trial 1 week after the 4th trial for the evaluation of memory acquisition and retention. The latency time of stay in the light room

from light-on was recorded following experience of electric shock (1 mA for 2 sec) in the dark compartment. Water-maze trials were performed in a circular water bath filled with water maintained at 22±2° C. The bath was divided into 4 quadrants and a hidden escape platform (10 cm in diameter) was submerged in the center of one quadrant. The rats were trained to learn to find the hidden platform, based on several cues external to the maze. Three trials were conducted on each day with 5-min intervals for the 4 consecutive days followed by 5th performance 1 week after the 4th day trial. The mean time spent to escape onto the platform was recorded.

Example 7

Acetylcholine Analysis in CSF

The rats were sacrificed at the end of learning/memory testing, and cerebrospinal fluid (CSF) was collected to analyze acetylcholine (ACh) contents. ACh concentration in CSF was measured with the Amplex Red acetylcholine/acetylcholinesterase assay kit (Molecular Probes) according to the manufacturer's instructions.

Example 8

Immunohistochemistry in Brain Sections

The rat brains were perfusion-fixed with 4% paraformaldehyde solution and post-fixed for 48 hr, followed by cryoprotection in 30% sucrose for 72 hr. Coronal cryosections 30-μm thick were prepared and processed for double immunostaining of human nuclear matrix antigen (hNuMA) and ChAT, neurofilament protein (for neurons) or GFAP (for astrocytes) using antibodies specific for hNuMA (1:100, mouse monoclonal, EMD Biosciences, San Diego, Calif.), ChAT (1:100, rabbit polyclonal, Chemicon), NF-Mix (1:1, 000, rabbit polyclonal, Chemicon) or GFAP (1:1,000, rabbit polyclonal, Chemicon). Brain sections were incubated with primary antibodies overnight at 4° C. and with secondary antibodies conjugated with Alexa Fluor-488 or -594 (1:1,000, Molecular Probes) for 1 hr at RT.

Example 9

Statistical Analysis

Data are presented as means±sem. The statistical significance between group comparisons for behavioral data was determined by one-way analysis of variance (ANOVA) and two-way ANOVA. P-values<0.05 were considered to be statistically significant.

Example 10

Establishment of Human NSC Lines Expressing ChAT

Transfection of F3 human NSCs with human ChAT gene (FIG. 1A) was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, displaying expression of ChAT mRNA in F3.ChAT cells (FIG. 1B). The expression levels of ChAT mRNA and protein in F3.ChAT cells are significantly higher than those in F3 cells (FIGS. 1B and 1D). The F3.ChAT cells showed similar morphology to their parental F3 cells (FIG. 1C) and F3.ChAT cells were highly immunoreaction-positive for ChAT antibody (FIG. 1D).

Example 11

Transplantation of F3.ChAT Cells into the Rat Brain Pre-treated with AF64A

At 9 weeks after icy transplantation of F3.ChAT cells (1×10^6 cells/rat) in AF64A-challenged rats, it was confirmed that F3.ChAT cells were distributed diffusely all over the brain regions (FIGS. 2A to 2D). ChAT-immnuoreactivity was detected in hippocampus, thalamus, hypothalamus, cortices and septum. In order to confirm transplanted F3.ChAT cells express ChAT activity in vivo, the inventors performed double immunostaining using antibodies specific for human nuclear matrix antigen (hNuMA) and ChAT, and the results indicate that most of the huNuMA-positive F3.ChAT cells strongly express ChAT at 9 weeks post-transplantation (FIG. 2E). In addition, F3.ChAT cells double-positive for hNuMA and glial fibrillary acidic protein (GFAP) as well as double-positive for hNuMA and neurofilament (NF) were found indicating that F3.ChAT cells differentiate into both astrocytes and neurons (FIGS. 2F and 2G). The ChAT protein was detected in the F3.ChAT cells which were diffused and distributed all over the brain regions of rat AD model after transplantation. The injected F3.ChAT cells survived in vivo up to 9 weeks after transplantation (FIG. 2).

Example 12

Restoration of the Acetylcholine Concentration by the Transplantation of F3.ChAT Cells

AF64A is a choline analogue which is taken up only by the high-affinity choline transport system into cholinergic neurons, and causes alterations in ChAT mRNA expression and enzyme activity (15-17). Hence, AF64A administration was found to decrease the release of ACh, and thereby induce cognitive impairments including memory and learning deficits (16-18). Therefore, the inventors adopted AF64A animal model to match the concept of memory improvement by F3.ChAT transplantation. Only decrease by 25-30% of ACh levels leads to a severe memory loss not only in β-amyloid transgenic (Tg2576) mice (19), but also in AF64A-injected animals who revealed symptoms within 2 weeks, lasting longer than 11 weeks (16-18, 20).

Seven and 11 weeks after icy injection of cholinotoxin AF64A (3 nmol/rat), ACh concentration in CSF significantly decreased as compared with saline-administered control rats (FIGS. 3A and 3B). Such decreases in ACh levels were fully restored in AF64A brain receiving F3.ChAT cell transplantation 2 weeks after AF64A challenge, while in AF64A animals receiving control F3 cells ACh level remained low.

Because of the diffuse distribution of the transplanted NSCs, we analyzed ACh concentration in CSF rather than discrete brain regions. It is believed that mRNA expression (FIG. 1B) and protein production (FIG. 1D) of ChAT from F3.ChAT cells increased the ACh levels in CSF.

Example 13

Improvement of Learning and Memory Function in AD Animal by the Implantation of F3.ChAT Cells

Rats challenged with AF64A (v) displayed severe impairment of learning and memory functions as measured by both passive avoidance (FIGS. 4A and 4B) and Morris water-maze (FIGS. 4C and 4D) performances 2 weeks (prior to cell transplantation, data not shown), 6-7 weeks (4-5 weeks after cell

transplantation, FIGS. 4A and 4C) and 10-11 weeks (8-9 weeks after cell transplantation, FIGS. 4B and 4D) after AF64A injection as compared with normal rats (●).

Interestingly, AF64A rats receiving of F3.ChAT cells (♦) fully recovered the learning and memory functions at both 4-5 weeks and 8-9 weeks post-transplantation, whereas control F3 cells (■) were ineffective in doing so. [●, normal control; ▼, AF64A alone administration; ■, AF64A+F3; ♦, AF64A+ F3.ChAT in FIG. 4]

As demonstrated in the above Example 12, transplantation of F3.ChAT cells (1×10^6 cells/rat) fully restored the ACh level in the rat brain (FIG. 3). Thus, it is reasonably believed that learning and memory function of AF64A-treated rats were improved through the action of the implanted F3.ChAT cells and restored ACh level, comparable to normal animals (FIG. 4). By comparison, F3 parental cells slightly elevated CSF ACh content, but exerted a negligible effect on the memory function. F3 and F3.ChAT cells migrated to lesion sites, differentiated into neurons and astrocytes, and survived up to 9 weeks after transplantation (FIG. 2). We were unable to count the exact number of cells in brain sections, because of their diffuse and scattered distribution all over the brain. However, it was confirmed that most of the hNuMA-positive F3.ChAT cells expressed ChAT (FIG. 2), indicating that the transplanted F3.ChAT cells functioned in the new environment of host brain tissue, which was further supported by the increase ACh level in CSF (FIG. 3).

As shown in the present invention, the inventors demonstrated that the brain transplantation of human NSCs overexpressing ChAT in AF64A-induced AD model rats fully restored learning and memory function and ACh levels in the CSF, comparable to normal animal.

Having described a preferred embodiment of the present invention, it is to be understood that variants and modifications thereof falling within the spirit of the invention may become apparent to those skilled in this art, and the scope of this invention is to be determined by appended claims and their equivalents.

REFERENCES

1. Terry, R. D. and Davies, P., Annu. Rev. Neurosci. 3, 77 (1980).
2. Whitehouse, P. J. et al., Science 215, 1237 (1982).
3. Coyle, T. J. et al., Science 219, 1184 (1983).
4. K. Kasa, P. et al., Prog. Neurobiol. 52, 511 (1997).
5. Terry, A. V. and Buccafusco, J. J., Pharmacol. Exp. Ther. 306, 821 (2003).
6. Musial, A. et al., Curr. Med. Chem. 14, 2654 (2007).
7. Goldman, S., Nat. Biotechnol. 7, 862 (2005).
8. Lindvall, O. and Kokaia, Z., Nature 441, 1094 (2006).
9. Kim, S. U. and deVellis, J. J., Neurosci. Res. 87, 2183 (2009).
10. Blurton-Jones, M. et al., Proc. Natl. Acad. Sci. USA 106, 13594 (2009).
11. Lee, H. J. et al., Stem Cells 25, 1204 (2007).
12. Lee, S. T. et al., Brain 131, 616 (2008).
13. Yasuhara, T. et al., J. Neurosci. 26, 12497 (2006).
14. Hwang, D. H. et al., Gene Ther. 16, 1034 (2009).
15. Fan, Q. I. and Hanin, I., Neurochem. Res. 24, 15 (1999).
16. Yamazaki, N. et al., Psychopharmacology (Berl.) 103, 215 (1991).
17. Bessho, T. et al., J. Neural Transm. 115, 1019 (2008).
18. Abe, E. et al., Br. J. Pharmacol. 108, 387 (1993).
19. Tsai, K.-J. et al., J. Exp. Med. 204, 1273 (2007).
20. Fisher, A. et al., J. Pharmacol. Exp. Ther. 222, 140 (1982).
21. Takashina, K. et al., J. Neural Transm. 115, 1027 (2008).
22. Shimato, S. et al., Gene Ther. 14, 1132 (2007).
23. Xuan, A. G. et al., Neurosci. Lett. 440, 331 (2008).
24. Wu, S. et al., Pathobiology 75, 186 (2008).
25. Srivastava, N. et al., Int. J. Dev. Neurosci. 27, 103 (2009).
26. Beck, K. D. et al., Nature 373, 339 (1995).
27. Schbitz, W. R. et al., Stroke 31, 2212 (2000).
28. Kim, S. U., Neuropathology 24, 159 (2004).
29. Matsuo, A. et al., Neurochem. Int. 46, 423 (2005).

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What is claimed is:

1. A method for treating Alzheimer's disease, comprising:

administering to a subject suffering from Alzheimer's disease, wherein the subject exhibits a decreased expression of acetylcholine, a therapeutically effective amount of a pharmaceutical composition comprising transfected HB1.F3 human neural stem cells (NSCs) in a manner that the transfected NSCs stably express human choline acetyltransferase (ChAT) in a brain tissue of the subject,

wherein the transfected HB1.F3 human NSCs are transfected with a retroviral vector comprising a polynucleotide encoding human ChAT including the amino acid sequence of SEQ ID NO:1, a human cytomegalovirus (CMV) promoter which is operatively linked to the polynucleotide encoding human ChAT, and a SV40 polyadenylation signal sequence,

wherein the NSCs are immortalized by an introduction of a retroviral vector containing v-myc oncogene, and

wherein the method restores the acetylcholine level of the subject comparable to a normal acetylcholine level and improves learning and memory function of the subject when compared to a subject that was administered with non-transfected HB1.F3 NSCs.

2. The method of claim 1, wherein the retroviral vector comprising a polynucleotide encoding human ChAT is pLPCX.

3. The method of claim 1, wherein the method comprises administering about 1×10⁶ NSCs.

4. The method of claim **1**, wherein the pharmaceutical composition further comprises one or more pharmaceutically acceptable carriers selected from solvents, dispersion media, coatings, antibacterial and antifungal agents, and isotonic and absorption delaying agents.

5. The method of claim **1**, wherein the pharmaceutical composition is administered by injection.

6. The method of claim **1**, wherein the pharmaceutical composition is administered by intracerebroventricular administration.

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